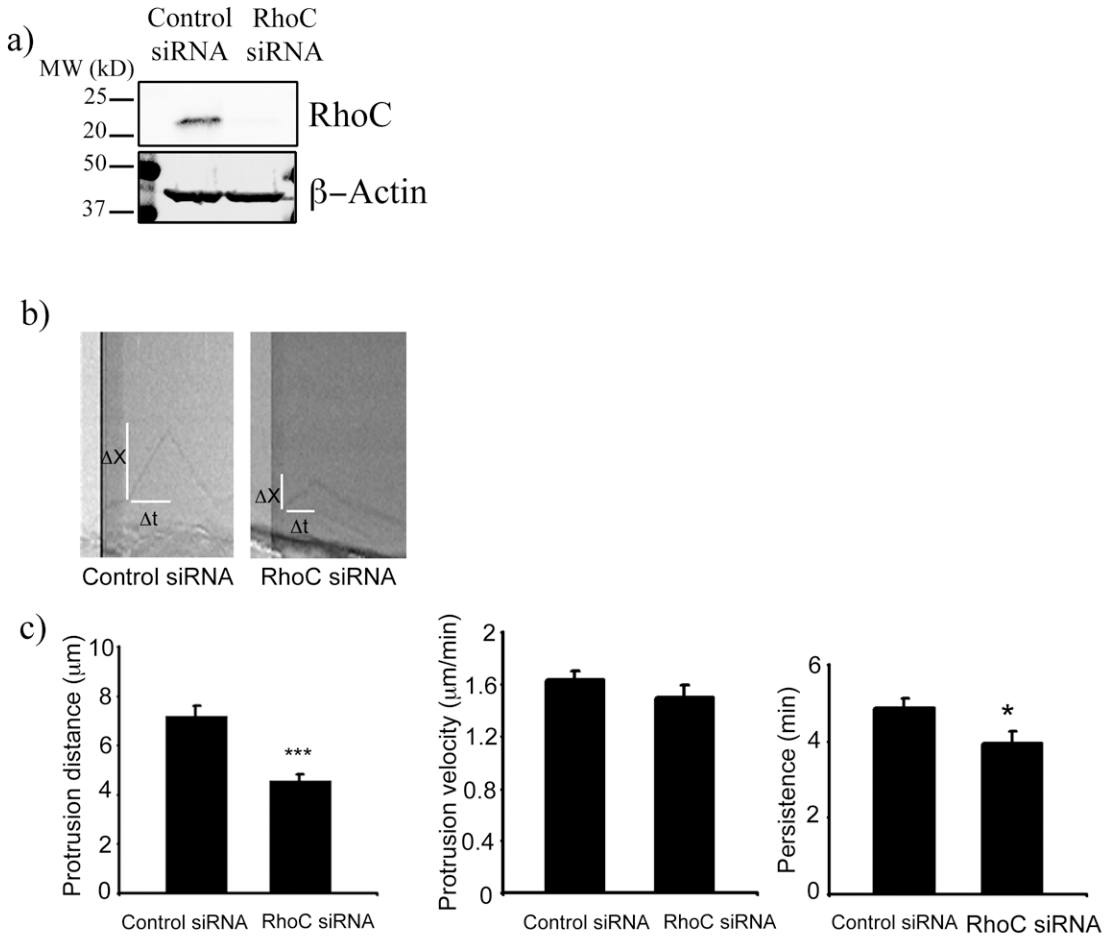


Fig. S1. RhoC is important for protrusions dynamics. a) Western blot of whole-cell lysates from MTLn3 transfected with control or RhoC siRNA and blotted for RhoC and β-actin. b) Representative kymographs of MTLn3 cells transfected with control or RhoC siRNA. Kymographs were generated by drawing a constant line in the stacked time series and the protrusion was followed over time. Δt=persistence of protrusions, ΔX=distance of protrusions, and ΔX1/Δt1=protrusion velocity (rate). c) Quantification of the dynamics of protrusions as measured by kymographs. Number of cells analyzed: control (22 cells), and RhoC siRNA (30 cells).

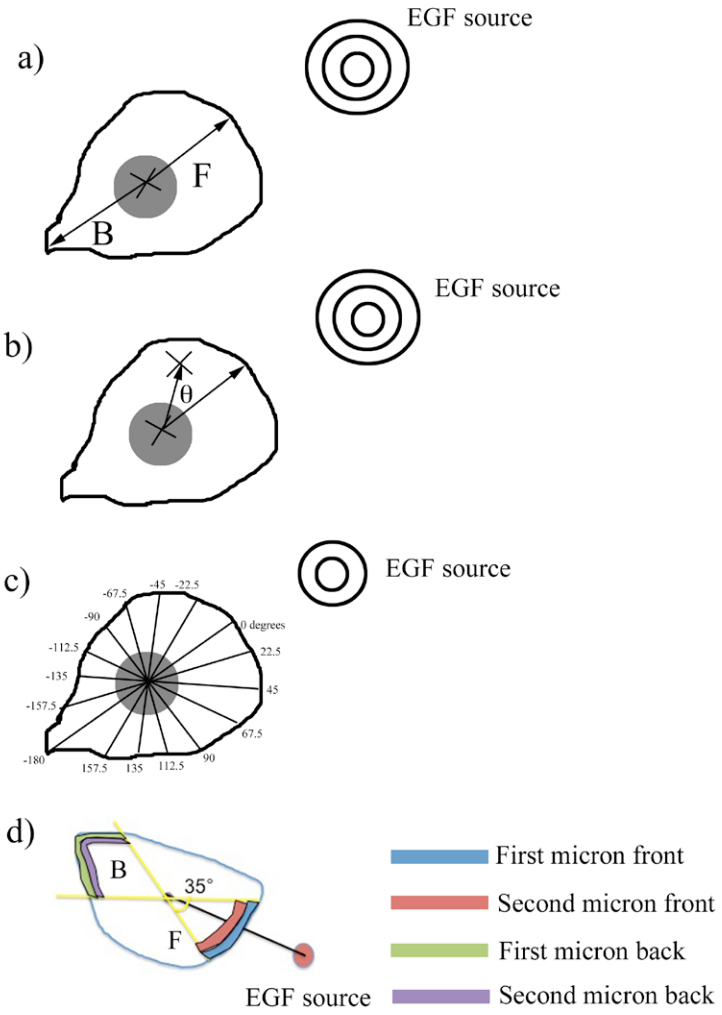


Fig. S2. Cartoon illustrating the methodology of the pipette experiments. a) Cartoon illustration showing front protrusion (F), which is the measurement of membrane protrusion along a line drawn from the centroid of the cell to the tip of the micropipette and back protrusion (B), which is the measurement of membrane protrusion along a line starting from the centroid and forming a 180° angle with the front line (Mouneimne et al., 2006). This analysis was used in Fig. 1D. b) Cartoon definition of angle θ of cell direction relative to the micropipette upon stimulation with EGF (θ is the angle between the motility path followed by the cell and the line formed by the initial centroid and pipette tip). This analysis was used in Fig. 1E. c) Cartoon representation showing the 16 lines dividing a stimulated cell into 16 sectors of 22.5° each. Concentric circles in a,b and c represent the EGF source. This analysis was used in Fig. 1F). d) Cartoon definition of the areas where RhoC activity was measured relative to the tip of the micropipette filled with EGF. This analysis was used in Fig. 2E).

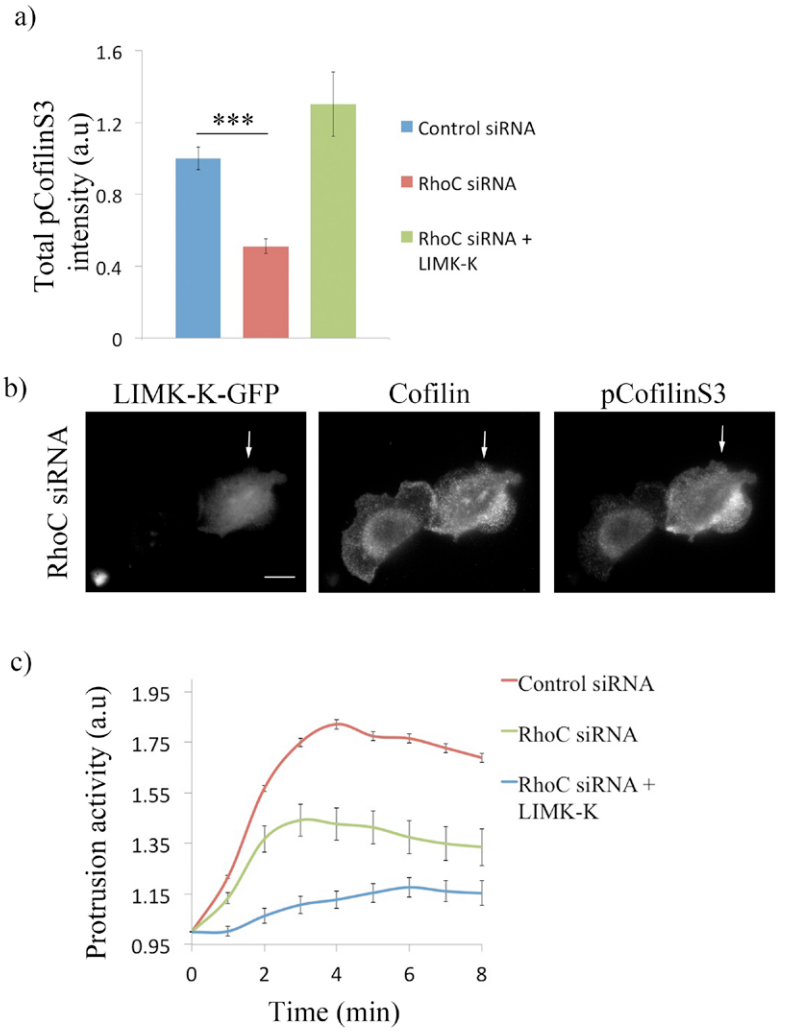


Fig. S3. Active LIMK (LIMK-K) rescues pCofilinS3 levels in cells depleted of RhoC. a) Quantification of total pCofilinS3 fluorescence intensity in the whole cell of control siRNA, RhoC siRNA and RhoC siRNA expressing LIMK-K (constitutively active) MTLn3 cells. Control siRNA=35, RhoC siRNA=29, RhoC siRNA+LIMK-K=17. Values are normalized to control siRNA. b) Representative images of RhoC siRNA depleted cells and expressing LIMK-K-GFP MTLn3 cells stained for cofilin and pCofilinS3. White arrows point towards the cell expressing LIMK-K-GFP. c) Quantification of protrusive activity in response to EGF in control, RhoC siRNA-treated cells and RhoC siRNA-treated cells expressing LIMK-K. Membrane protrusion is standardized to time 0. Number of cells analyzed: control siRNA=26 cells, RhoC siRNA=28, RhoC siRNA+LIMK-K=14. ***P<0.001. Error bars represent s.e.m. Scale bar represents 10 μm.

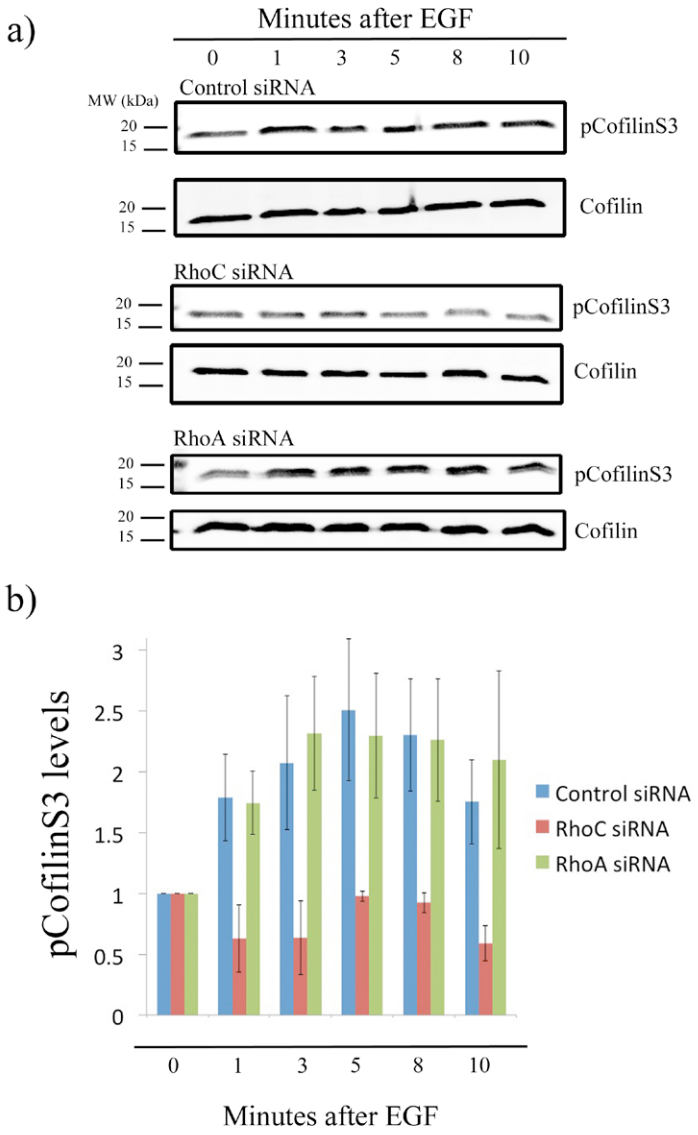


Fig. S4. RhoC but not RhoA regulates cofilin phosphorylation at serine 3. Western blots (a) and quantification (b) of MTLn3 lysates from control, RhoC or RhoA siRNA treated cells blotted for cofilin and pCofilinS3. Cells were starved for 3 hr and stimulated with 5 nM EGF for 0, 1, 3, 5, 8 or 10 minutes. *n*=at least 2 independent experiments. pCofilinS3 protein levels are standardized to total cofilin.

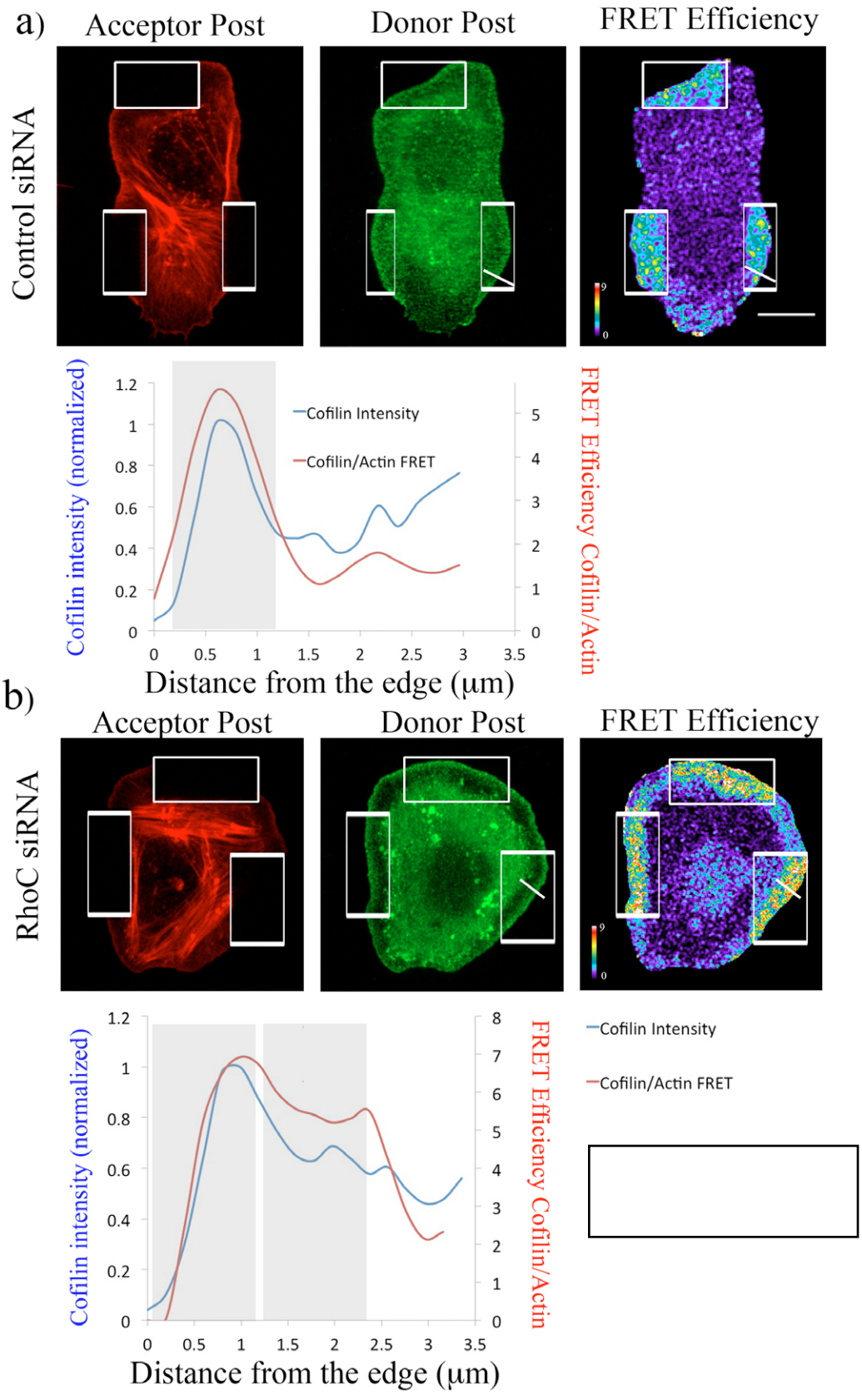


Fig. S5. Acceptor photobleaching images of cofilin/actin FRET from control and RhoC siRNA treated cells. a) Representative images of control siRNA-treated MTLn3 cells EGF-stimulated, fixed and labeled for cofilin (donor; Alexa 488) and F-actin (acceptor; Rhodamine). Acceptor post-bleaching, donor post-bleaching and FRET efficiency image are shown. Cofilin and F-Actin intensity profiles along the indicated line in FRET efficiency image are shown. Scale bars represent 10 μm. b) Representative images of RhoC siRNA-treated MTLn3 cells EGF-stimulated, fixed and labeled for cofilin (donor; Alexa 488) and F-actin (acceptor; Rhodamine). Acceptor post-bleaching, donor post-bleaching and FRET efficiency image are shown. Cofilin and F-Actin intensity profiles along the indicated line in FRET efficiency image are shown. Scale bars represent 10 μm.

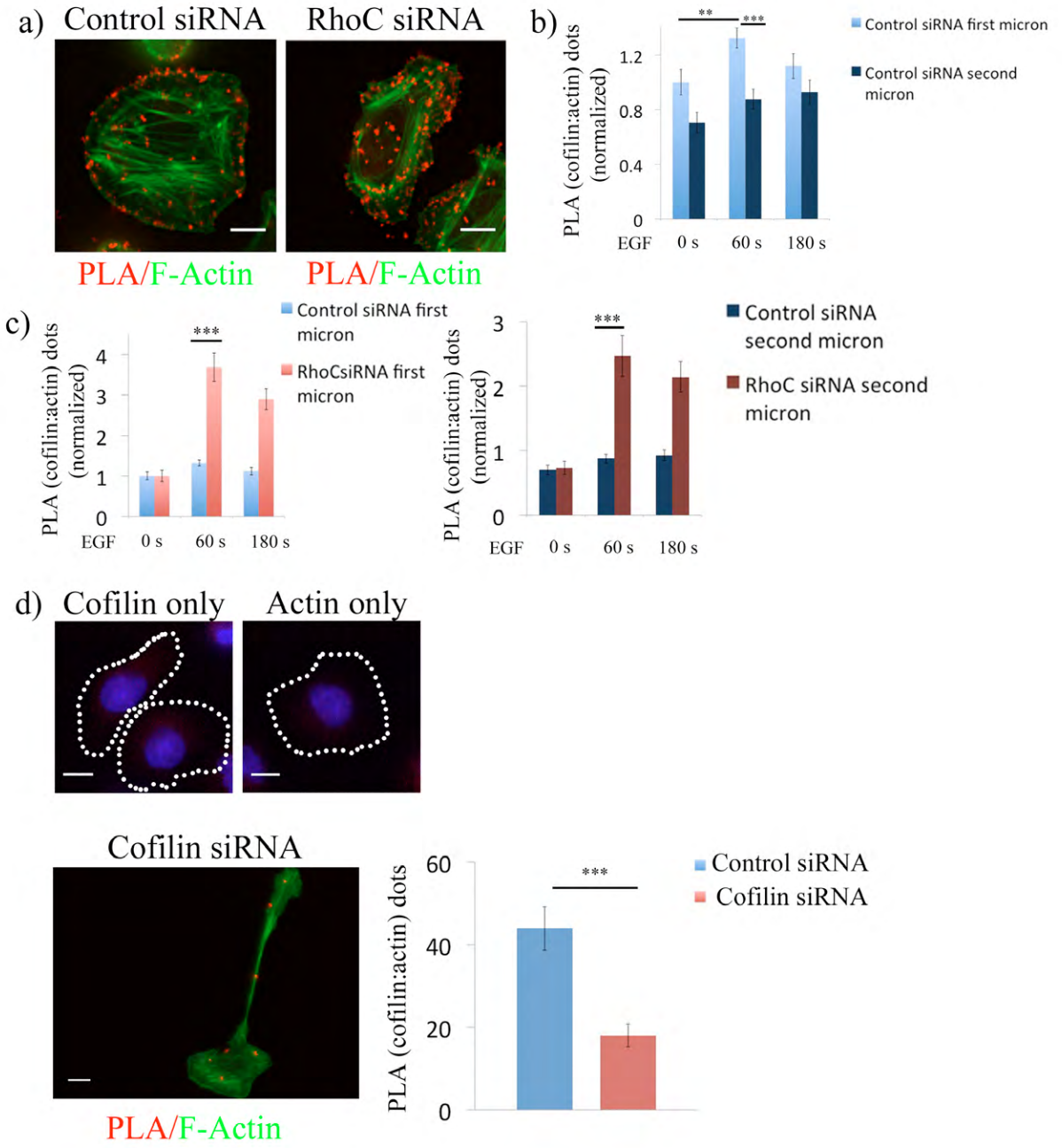


Fig. S6. RhoC spatially confines cofilin/actin interaction measured by PLA (proximity ligation assay). a) Representative images of the EGF-stimulated control and RhoC siRNA-treated MTLn3 cells where PLA was performed with specific antibodies against cofilin and actin, are shown. PLA dots are shown in red, F-actin staining (green) is also shown. b) Graphs represent PLA measurements calculated in: an area of one micron width right at the cell edge and in an area one micron width and one micron behind the cell edge for control MTLn3 cells unstimulated, and stimulated with EGF for 1 or 3 minutes. Data are normalized to time 0. The data shown represent the average of >25 cells per condition. Control cells showed an increase in the cofilin/actin interaction at the leading edge. c) Graphs represent PLA measurements calculated in: an area of one micron width right at the edge (left graph) or in an area one micron in width and one micron behind the cell edge (right graph), for control and RhoC siRNA-treated MTLn3 cells unstimulated, and stimulated with EGF for 1 or 3 minutes. In RhoC depleted cells distribution of the cofilin/actin interaction is no longer restricted to the first micron of the leading edge. Data are normalized to time 0. The data shown represent the average of >25 cells per condition. d) Cells stained with one antibody only (cofilin or actin) do not show any PLA dots. DAPI is shown in blue. Dotted lines represent the cell edge. e) Knockdown of cofilin significantly decreases the amount of PLA-Cofilin/Actin dots. Representative image of MTLn3 knockdown for cofilin where PLA was performed. PLA dots are shown in red, F-actin staining (green) is also shown. Graph shows the quantification of whole cell PLA dots in control or cofilin knockdown siRNA-treated cells stimulated with EGF for 1 minute. Scale bars represent 10 μm. The data shown represent the average of >10 cells per condition. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent s.e.m.

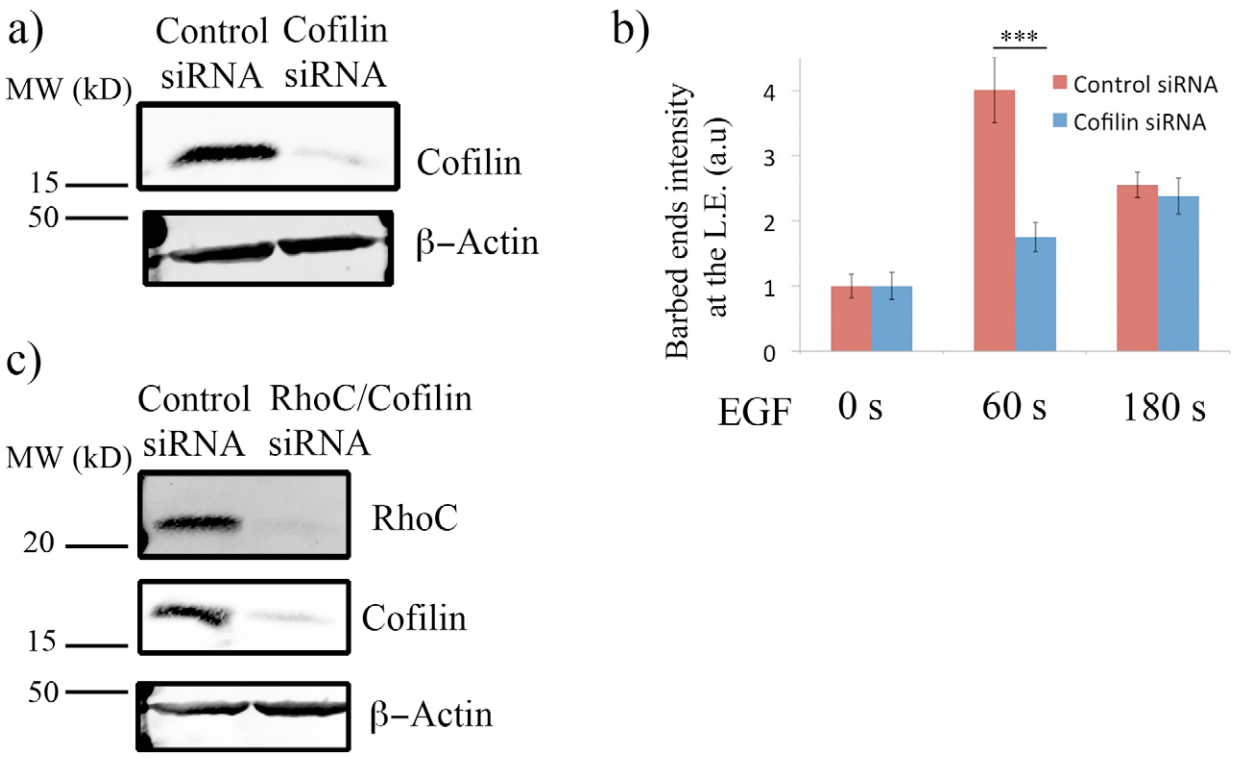


Fig. S7. Cofilin knockdown suppresses the early barbed end transient. a) Western blots of MTLn3 lysates from control and Cofilin siRNA treated cells blotted for Cofilin and β-actin. b) Quantification of barbed end intensity in response to EGF at lamellipodia in control and cofilin siRNA-treated MTLn3 cells. The data shown represent the average of >10 cells per condition. c) Western blots of MTLn3 lysates from control and RhoC/Cofilin siRNA treated cells blotted for Cofilin, RhoC and β-actin. ***P<0.001. Error bars represent s.e.m.

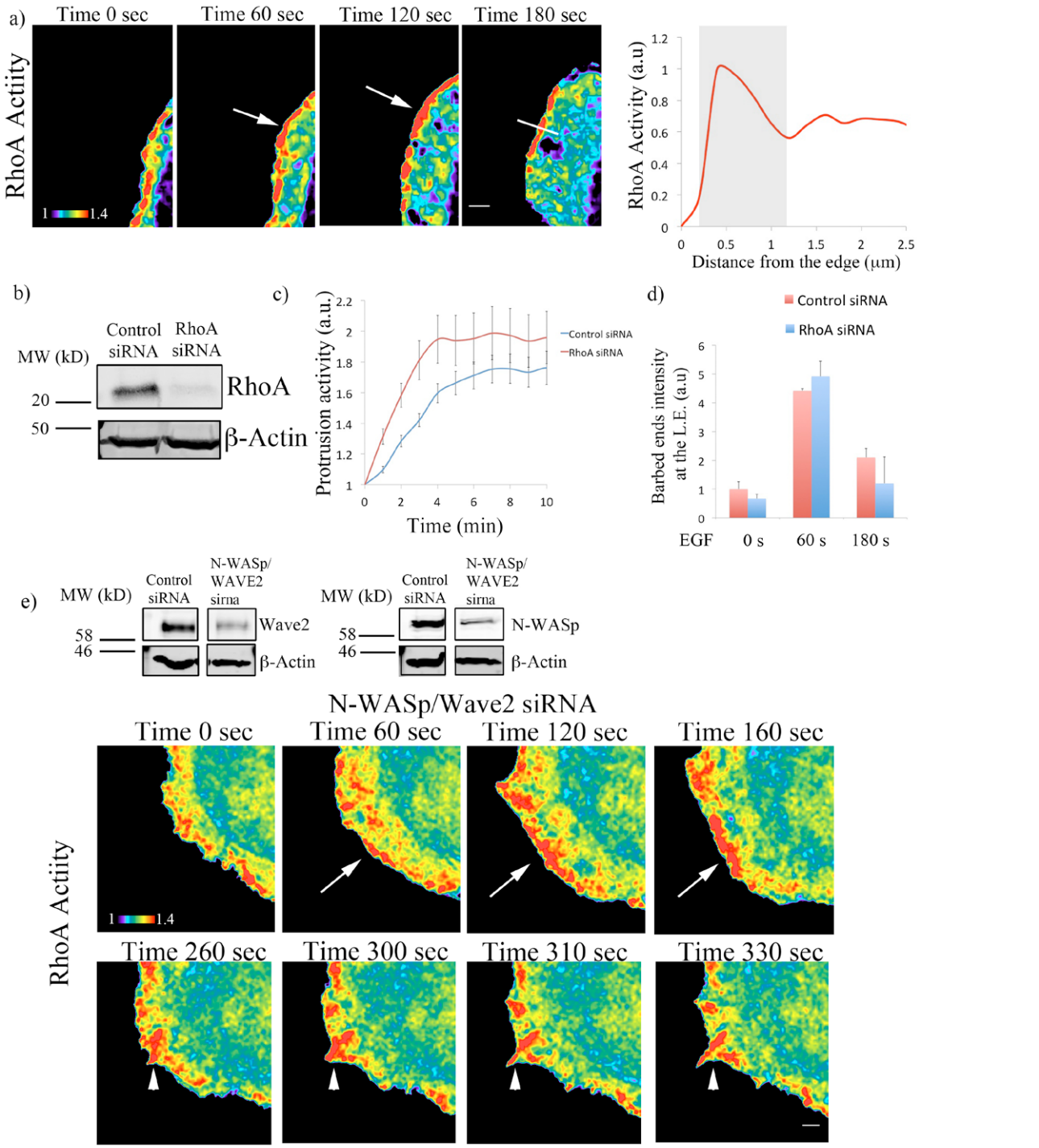


Fig. S8. RhoA activation and its role during EGF-stimulated protrusions. a) Still images from a time-lapse movie of MTLn3 expressing RhoA biosensor after EGF stimulation. White arrow points to area of high RhoA activation. Time points shown are the time after EGF was added. RhoA activity profiles along the indicated white line in the 180 sec image. Grey square highlight the first micron at the cell edge. b) Western blots of MTLn3 lysates from control or RhoA siRNA treated cells blotted for RhoA and β-actin. c) Quantification of protrusive activity in response to EGF in control and RhoA siRNA-treated cells. Membrane protrusion is standardized to time 0. Number of cells analyzed: control siRNA=29 cells, RhoA siRNA=25. d) Quantification of barbed end intensity in response to EGF at lamellipodia in control siRNA-treated MTLn3 cells and RhoA siRNA-treated MTLn3 cells. *n*=2 independent experiments with more than 20 cells per group. e) Western blots of MTLn3 lysates from control and NWASp/ Wave2 siRNA treated cells blotted for NWASp, Wave2 and β-actin. Still images from a time-lapse movie of MTLn3 depleted for N-WASp/Wave2 and expressing RhoA biosensor after EGF stimulation. White arrows point to areas of high RhoA activation. White arrowheads point to areas of high RhoA activity in filopodia-like protrusions. Time points shown are the time after EGF was added. Scale bars represent 2 μm.

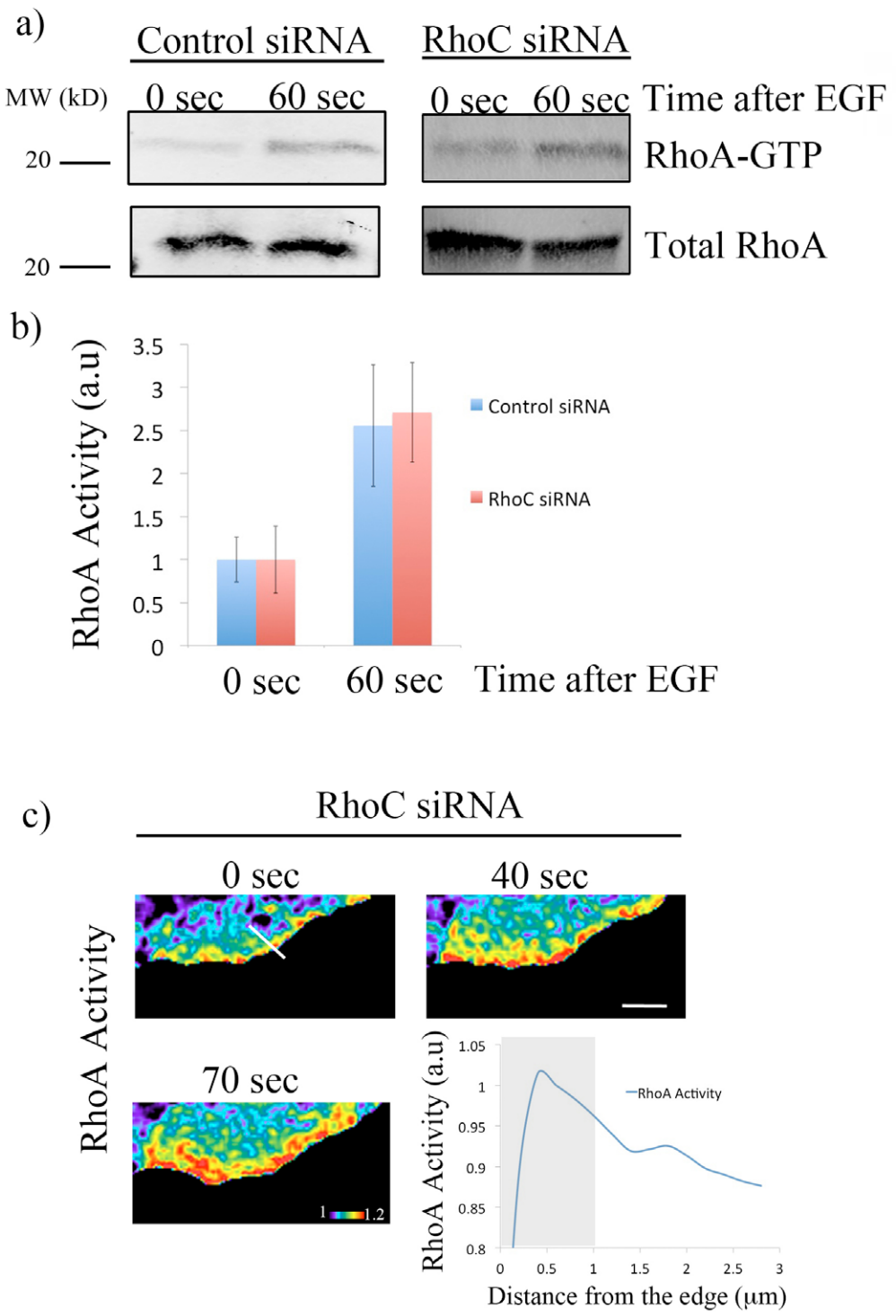


Fig. S9. RhoC depletion does not affect RhoA activation and distribution. a) Representative western blots of GST-RBD pulldowns showing RhoA activation. MTLn3 cells treated with control or RhoC siRNA were stimulated with EGF for 0 and 60 seconds. The amount of total RhoA in cell lysates is shown. b) Quantification of RhoA-GTP in control and cells depleted of RhoC from western blot. n=4 independent experiments. c) Still images from a time-lapse movie of MTLn3 depleted of RhoC expressing RhoA biosensor after EGF stimulation. RhoA activity profiles along the indicated white line in the 0 sec image. Grey square highlights the first micron at the cell edge. Scale bars represent 2 μm.

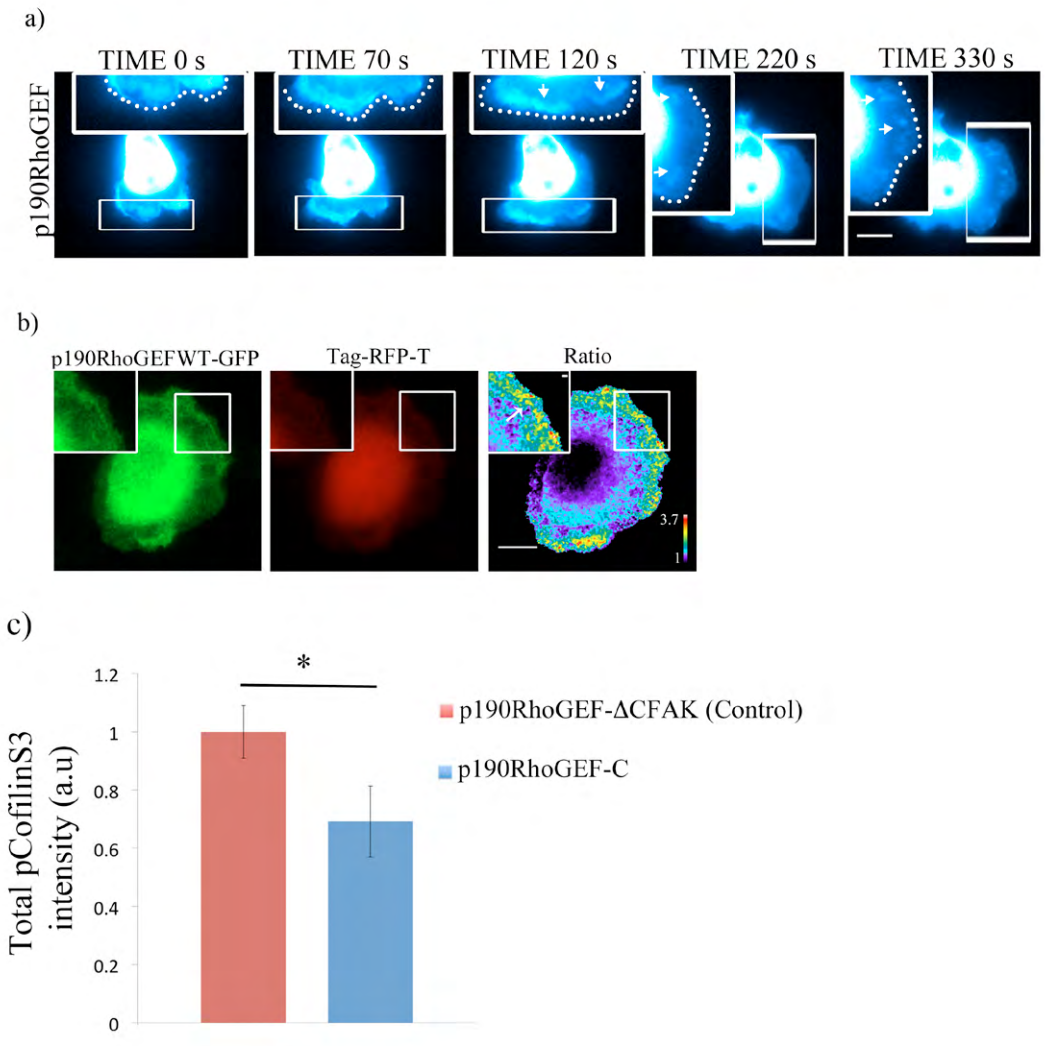


Fig. S10. p190RhoGEF localization during EGF stimulated protrusions. a) Still images from a time-lapse of MTLn3 cells expressing p190RhoGEF-GFP at different time points after EGF stimulation. Dotted lines in the close-up image represent the cell edge. White arrows point to areas of p190RhoGEF-GFP accumulation. b) Representative images of MTLn3 cells expressing p190RhoGEF WT-GFP and mTag-RFP-T (as a volume marker). A ratio image of the p190RhoGEF image divided by mTag-RFP-T image is shown in a pseudocolor scale. White arrows indicate accumulation of p190RhoGEF WT-GFP. Scale bars represent 10 μm. Insert scale bars represent 1 μm. c) Quantification of total pCofilinS3 fluorescence intensity in the whole cell of MTLn3 expressing p190RhoGEF-CΔFAK (Control) or p190RhoGEF-C-mCherry. p190RhoGEF-CΔFAK=19 cells, p190RhoGEF-C-mCherry=9 cells. Values are normalized to control (p190RhoGEF-CΔFAK). *P<0.05, Error bars represent s.e.m.

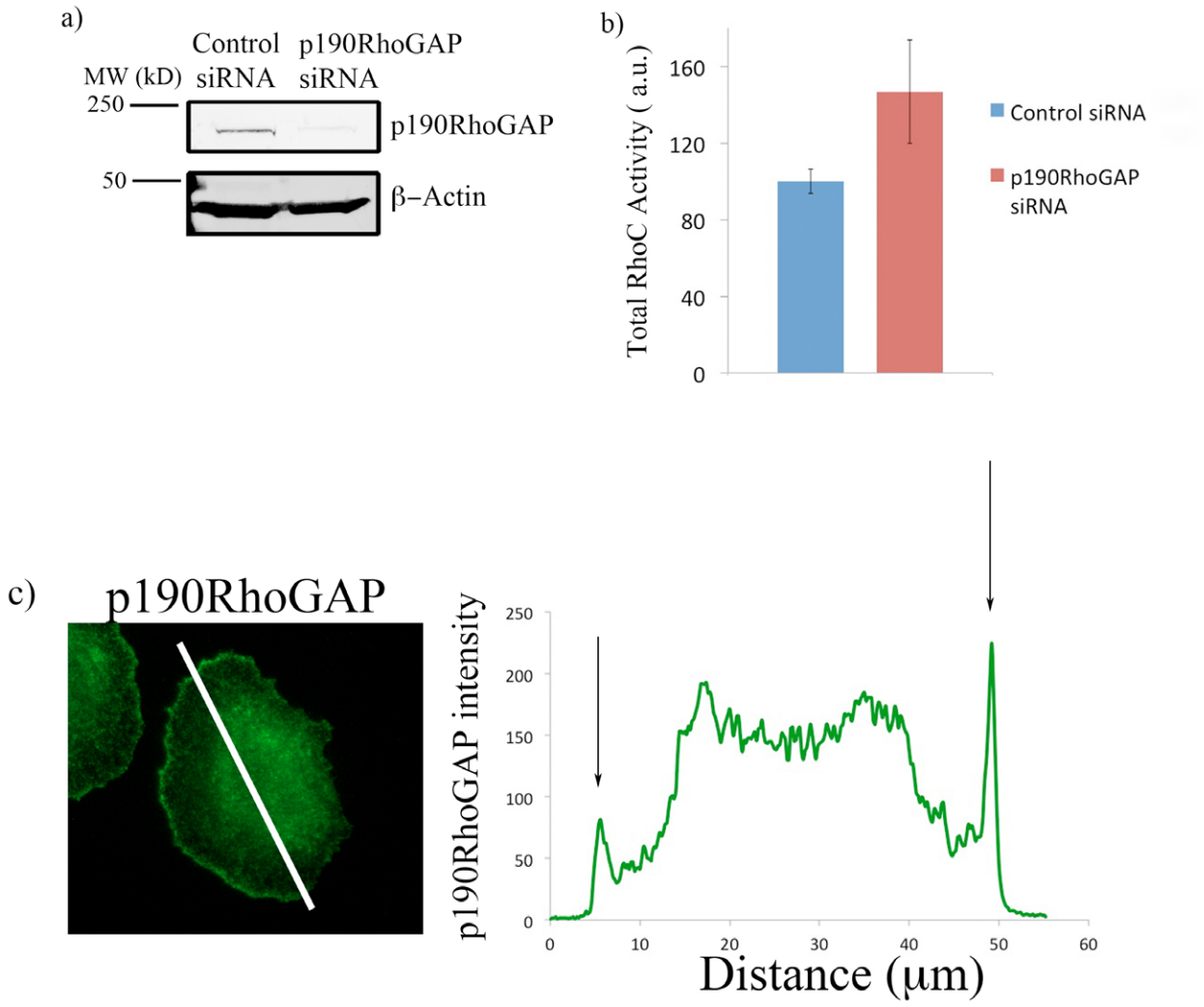


Fig. S11. p190RhoGAP depletion increases RhoC activity. a) Western blots of MTLn3 lysates from control and p190RhoGAP siRNA treated cells blotted for p190RhoGAP and β-actin. b) Quantification of RhoC activity (by using RhoC FLARE biosensor measurements) in the whole cell of control and p190RhoGAP siRNA-treated MTLn3 cells expressing RhoC biosensor. Values are normalized to control siRNA. Number of cells analyzed: control siRNA=16, p190RhoGAP siRNA=26. c) p190RhoGAP staining in MTLn3 cells. Linescan corresponds to the white line in the p190RhoGAP image. Arrows in the linscans point out to the edge of the cell.

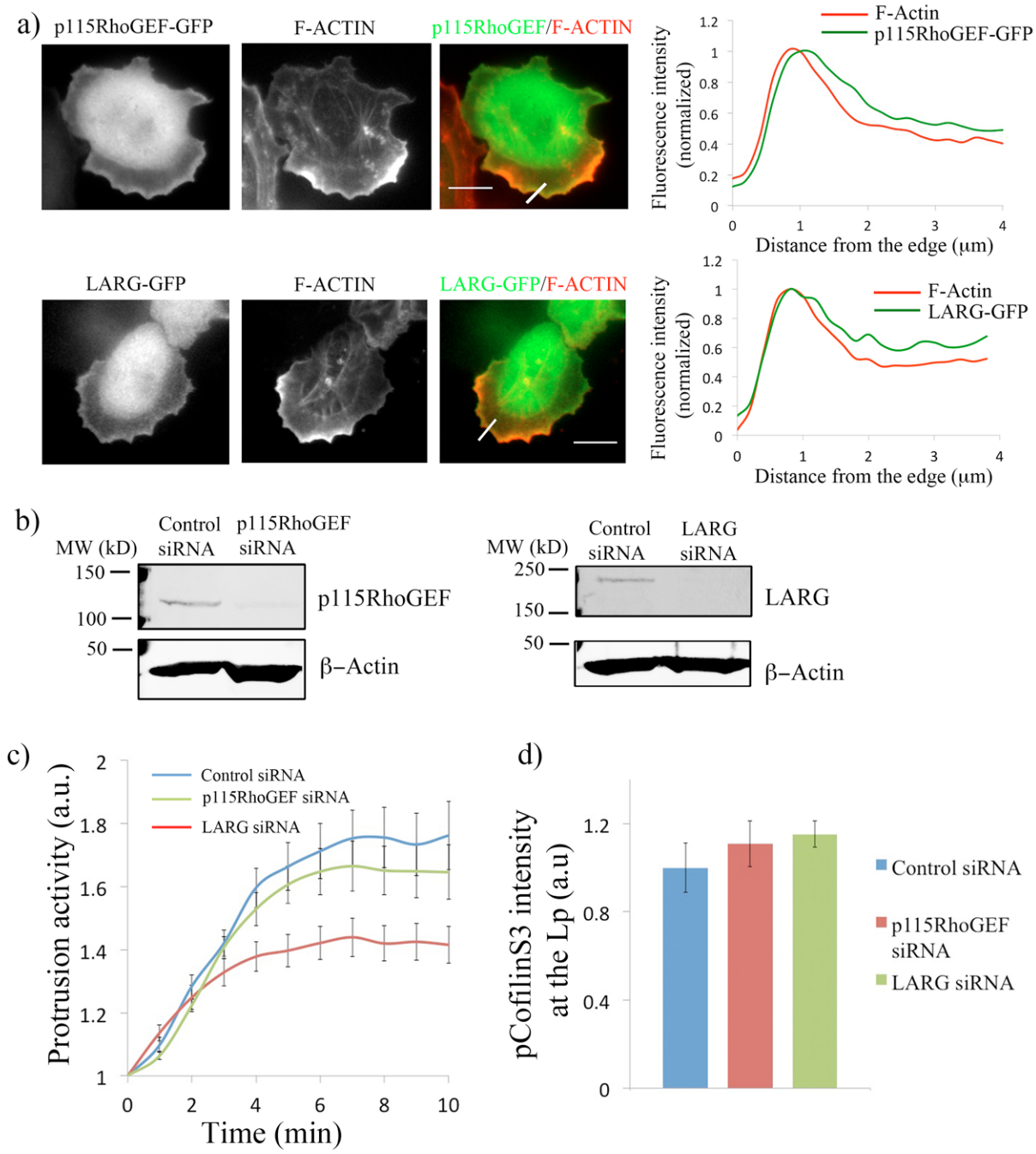


Fig. S12. Role of p115RhoGEF and LARG during lamellipodium protrusions and cofilin phosphorylation. a) Representative images of MTLn3 cells expressing p115RhoGEF-GFP or LARG-GFP and stained for F-Actin. p115RhoGEF WT-GFP, LARG-GFP and F-Actin intensity profiles along the indicated line in overlay image are shown. b) Western blot of whole-cell lysates from MTLn3 transfected with control, p115RhoGEF or LARG siRNA and blotted for p115RhoGEF, LARG and β-actin. c) Quantification of protrusive activity in response to EGF in control, p115RhoGEF and LARG siRNA-treated cells. Membrane protrusion is standardized to time 0. Number of cells analyzed: control siRNA=29 cells, p115RhoGEF siRNA=29 LARG=21 cells. d) Quantification of pCofilinS3 fluorescence intensity at the lamellipodium of control, p115RhoGEF and LARG siRNA MTLn3 cells stimulated with EGF. Values are normalized to control siRNA. Number of cells analyzed: control siRNA=22 cells, p115RhoGEf siRNA=24 LARG=28 cells. Scale bars represent 10 μm.

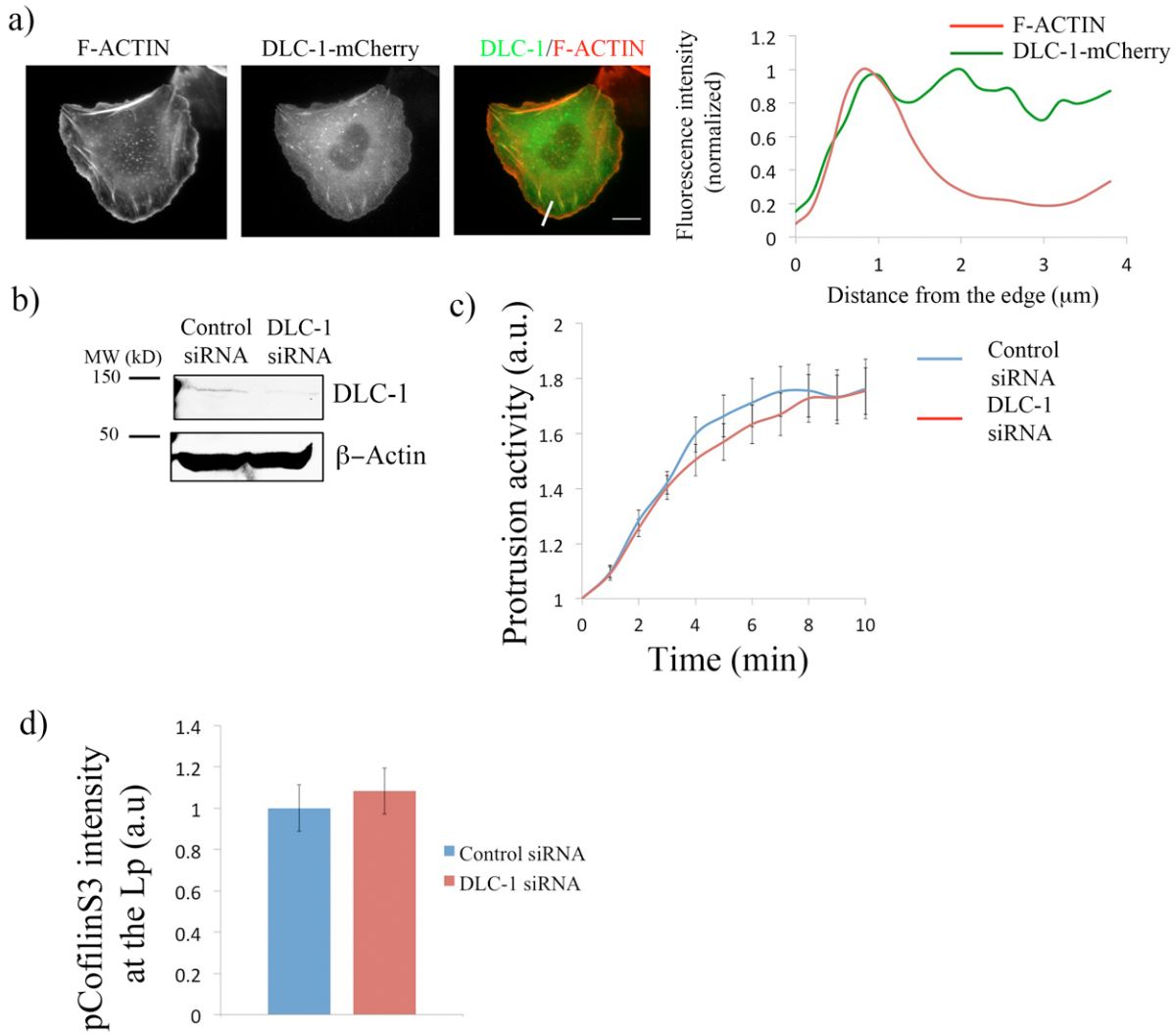


Fig. S13. DLC-1 depletion does not affect lamellipodium protrusion and cofilin phosphorylation. a) Representative images of MTLn3 cells expressing mCherry-DLC-1 and stained for F-Actin. mCherry-DLC1 and F-Actin intensity profiles along the indicated line in overlay image are shown. b) Western blot of whole-cell lysates from MTLn3 transfected with control or DLC-1 siRNA and blotted for DLC-1 and β-actin. c) Quantification of protrusive activity in response to EGF in control and DLC-1 siRNA-treated cells. Membrane protrusion is standardized to time 0. Number of cells analyzed: control siRNA=29 cells, DLC-1 siRNA=27. d) Quantification of pCofilinS3 fluorescence intensity at the lamellipodium of control and DLC-1 siRNA MTLn3 cells stimulated with EGF. Values are normalized to control siRNA. Number of cells analyzed: control siRNA=22 cells, DLC-1 siRNA=20. Scale bars represent 10 μm.

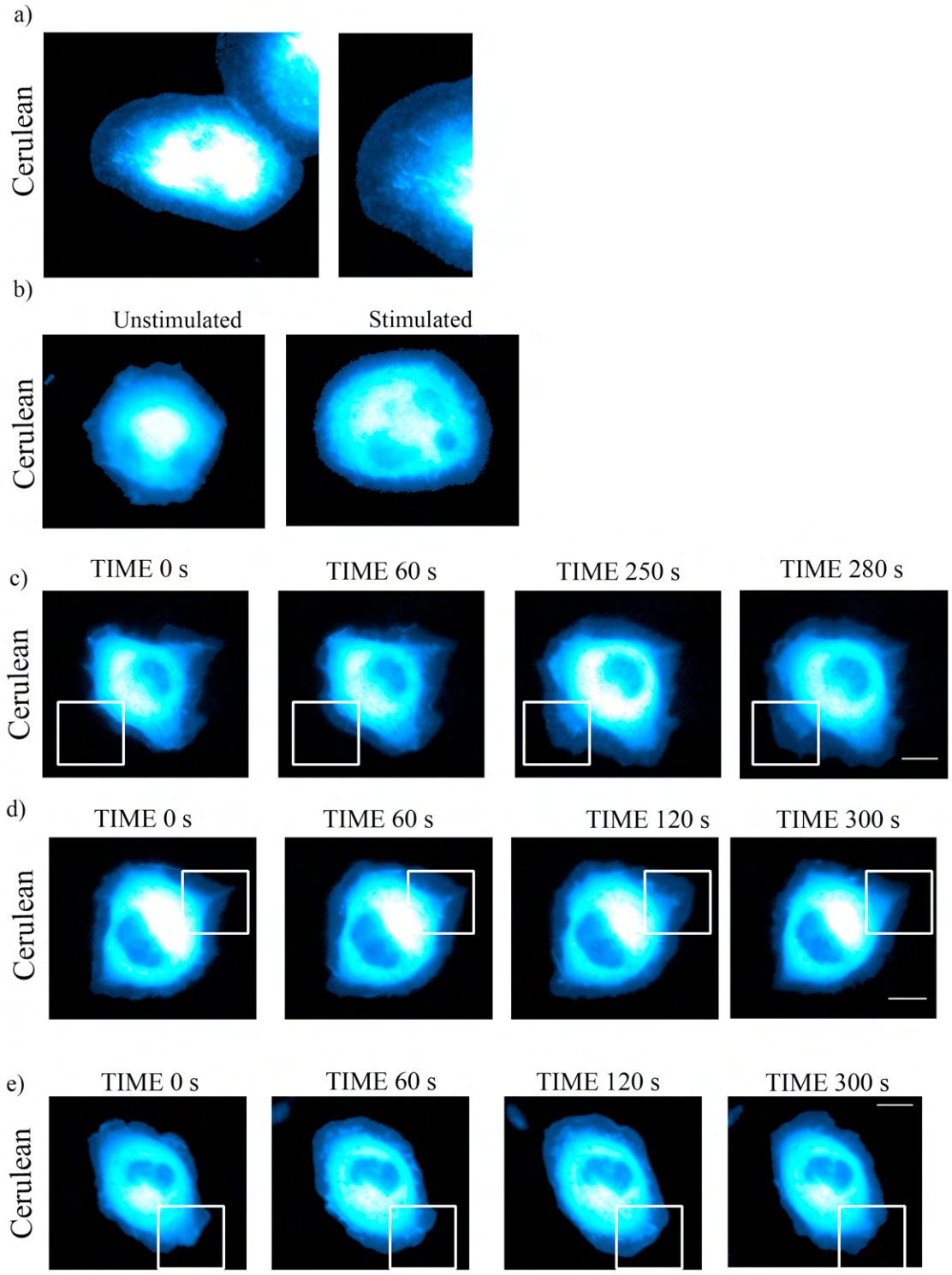


Fig. S14. Monomeric Cerulean images corresponding to the RhoC biosensor experiments. a) Images show mCerulean intensity corresponding to RhoC biosensor images from Fig. 2A. b) Images show mCerulean intensity corresponding to RhoC biosensor images from Fig. 2C. c) Images show mCerulean intensity corresponding to RhoC biosensor images from Fig. 5B. d) Images show mCerulean intensity corresponding to RhoC biosensor images from Fig. 5C. d) Images show mCerulean intensity corresponding to RhoC biosensor images from Fig. 5F.

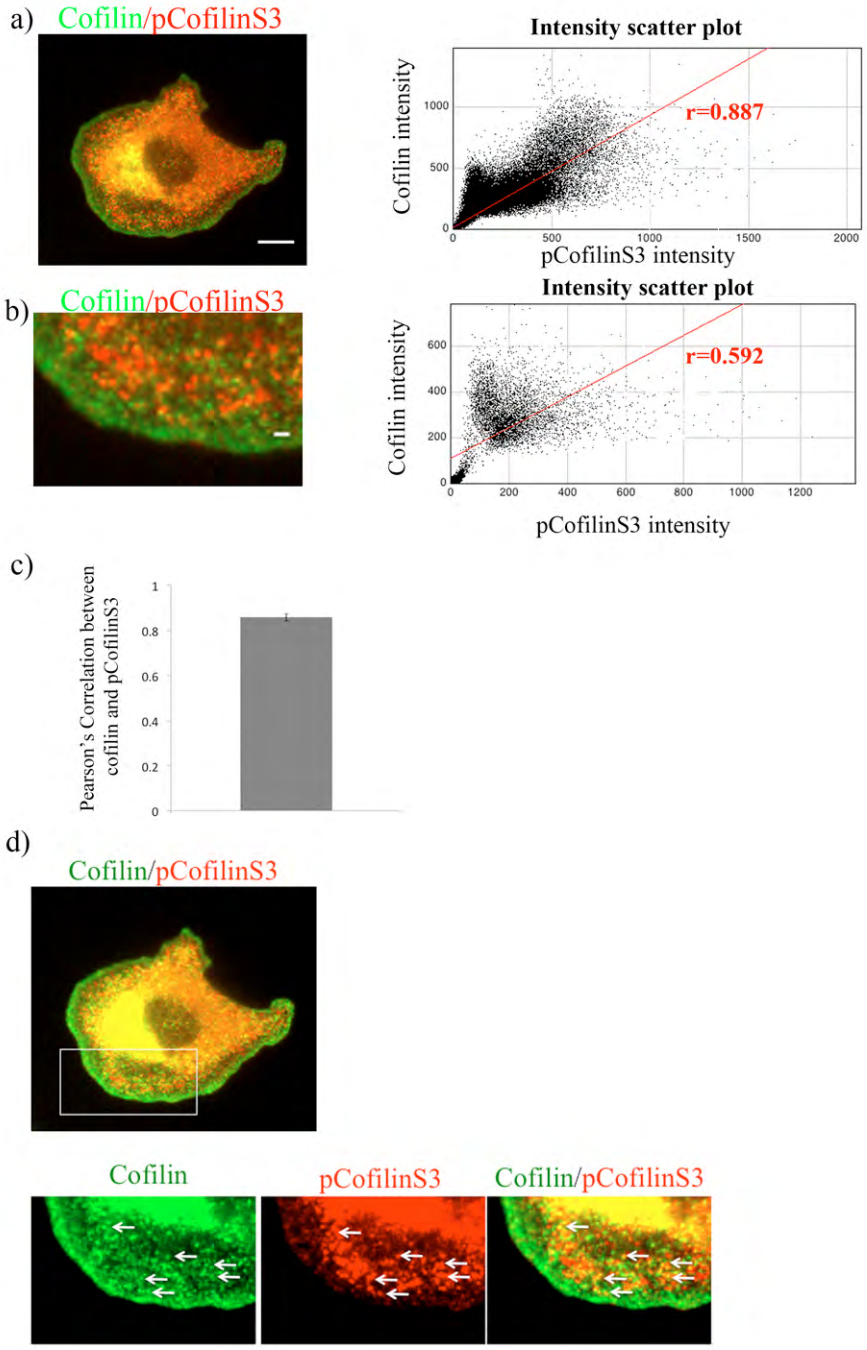


Fig. S15. Colocalization experiments between cofilin and pCofilinS3. a) MTLn3 staining for Cofilin and pCofilinS3 image corresponding to Fig. 3a and corresponding intensity scatter plot showing Pearson's correlation coefficient of r=0.887. b) Insert image corresponding to Fig. 3A and corresponding intensity scatter plot showing Pearson's correlation coefficient of r=0.592. c) Quantification of Pearson's correlation between cofilin and pCofilinS3 in the whole cell. *n*=9 cells. d) Images corresponding to Fig. 3A with increased brightness and contrast.

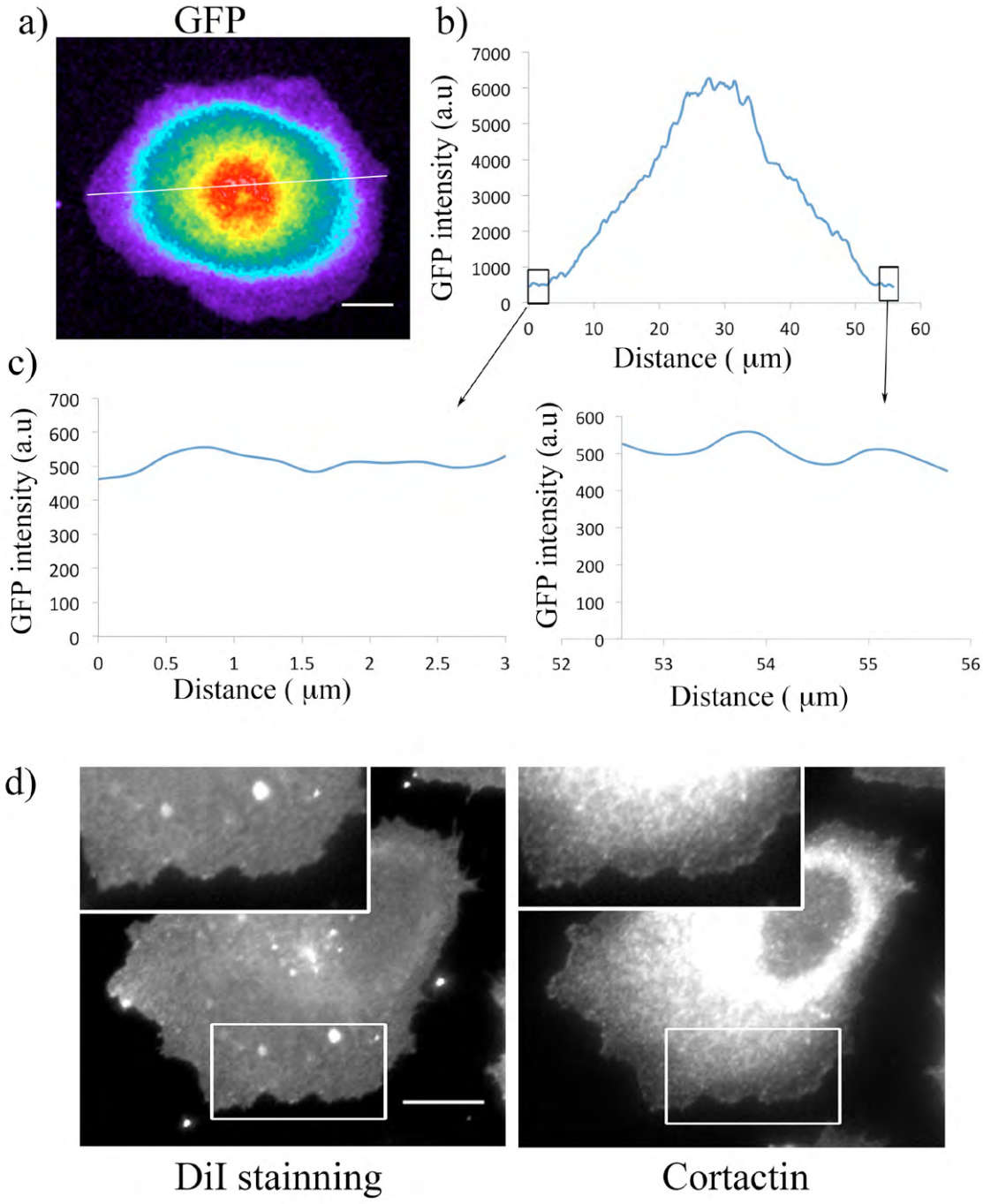


Fig. S16. MTLn3 cells display a flat lamellipodium with no ruffles. a) Representative image of the EGF-stimulated MTLn3 cell expressing GFP. b) Linescan profile along the white line in the image. c) Close up of the first three microns from the edge of the cells at the two ends of the white line in the GFP image showing no changes in thickness in the lamellipodium. d) DiI (lipophilic tracer) and cortactin staining of MTLn3 cells showing no ruffles or membrane folding at the lamellipodium.

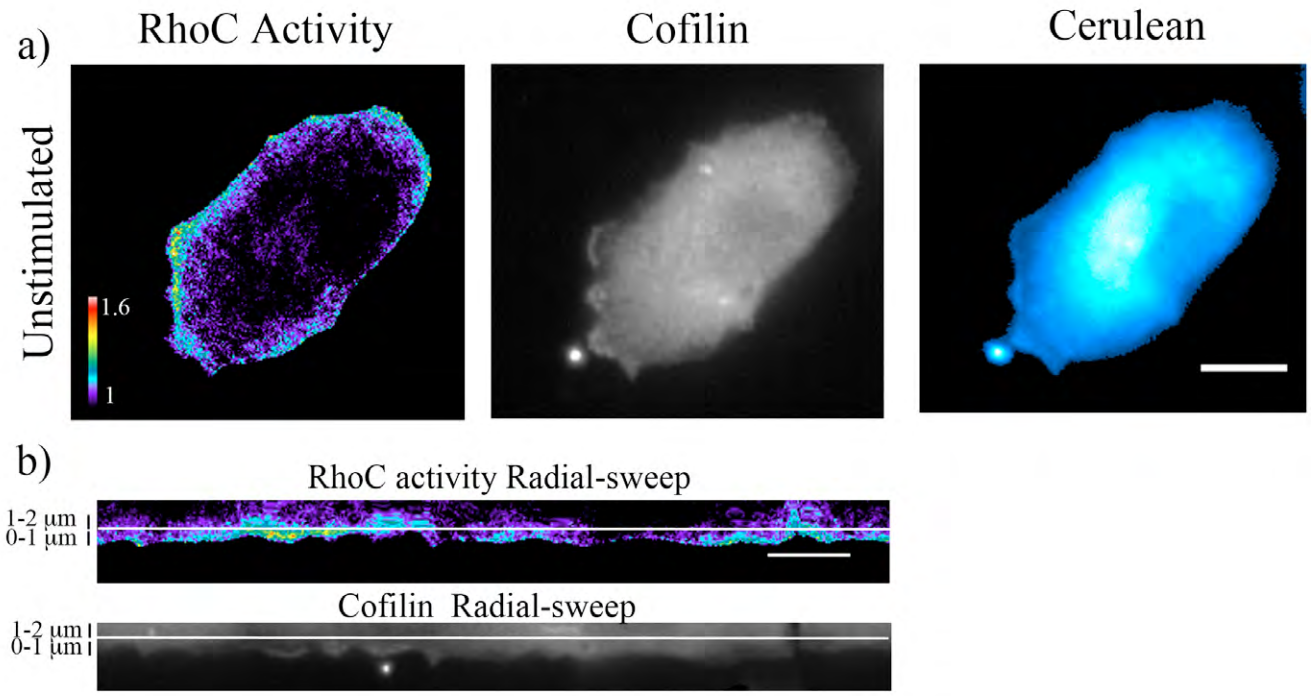
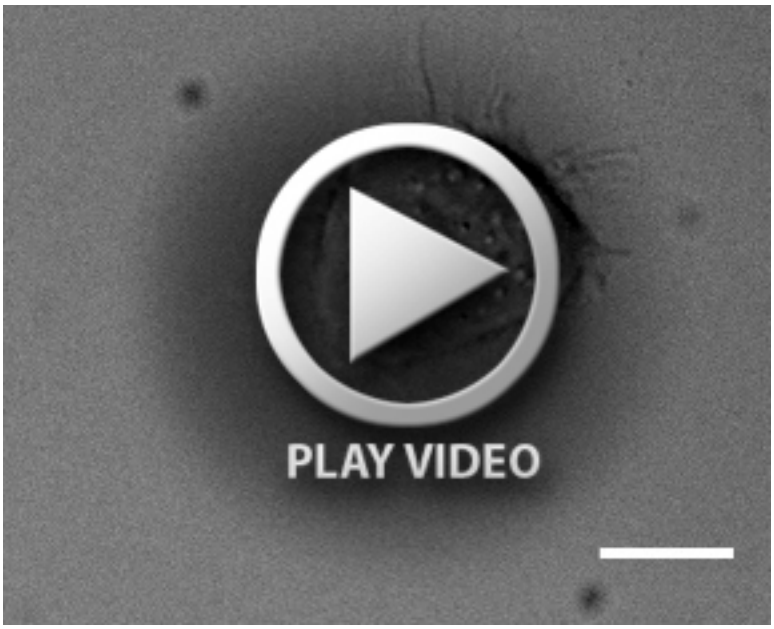


Fig. S17. RhoC activity in MTLn3 cells during starvation conditions. RhoC activity and staining of cofilin in MTLn3 cells expressing RhoC biosensor before stimulation with an EGF micropipette. RhoC activity and cofilin radial sweep is shown (this sweep is the outermost 4 µm strip of the cell). Spatial distances and horizontal line in the radial sweep images highlight the first and the second microns.

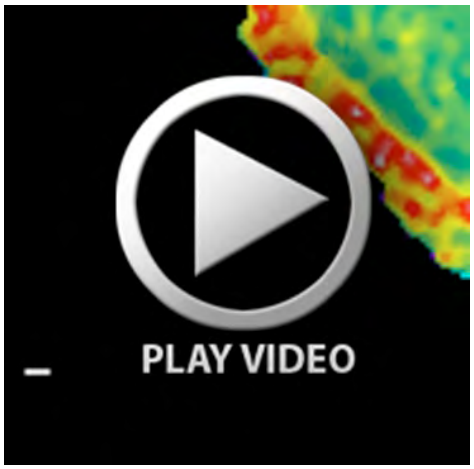
Movie 1. Phase images of MTLn3 cells plated on glass. MTLn3 cells were starved for 3 h and stimulated with 5 nM EGF at 37°C, EGF was added in the third frame. The images were taken at 20 s intervals. Movie corresponds to Fig. 1A Control siRNA. Total duration: 8 minutes. Scale bars represent 10 μm.



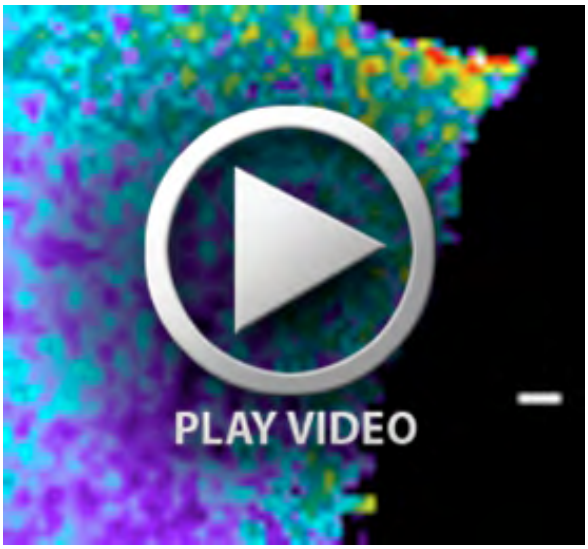
Movie 2. Phase images of RhoC siRNA-treated MTLn3 cells. MTLn3 cells were starved for 3 h and stimulated with 5 nM EGF at 37°C, EGF was added in the third frame. The images were taken at 20 s intervals. Movie corresponds to Fig. 1B RhoC siRNA. Total duration: 8 minutes. Scale bars represent 10 μm.

Movie 3. Phase images of MTLn3 cells plated on glass. MTLn3 cells were starved for 3 h and stimulated with an EGF-filled pipette at 37°C. The images were taken at 10 s intervals. Movie corresponds to Fig. 1C. Total duration: 10 minutes. Scale bars represent 10 μm.

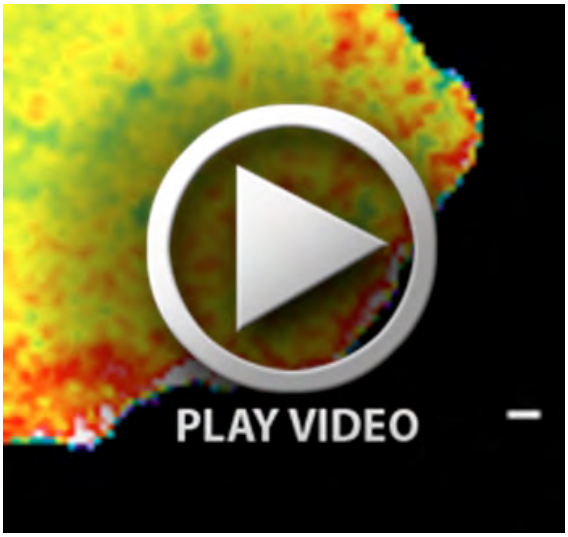
Movie 4. Phase images of RhoC siRNA-treated MTLn3 cells plated on glass. MTLn3 cells were starved for 3 h and stimulated with an EGF-filled pipette at 37°C. The images were taken at 10 s intervals. Movie corresponds to Fig. 1C. Total duration: 10 minutes. Scale bars represent 10 μm.



Movie 5. Ratio (FRET/CFP) images of MTLn3 cells. MTLn3 cells expressing the RhoC biosensor were starved for 3 h and stimulated with 5 nM EGF at 37°C (EGF was added in the first frame). The images were taken at 10 s intervals. Movie corresponds to the Fig. 5B. Total duration: 4.6 minutes. Black line and arrows highlight the areas of high RhoC activity behind the leading edge. Scale bars represent 1 μm.

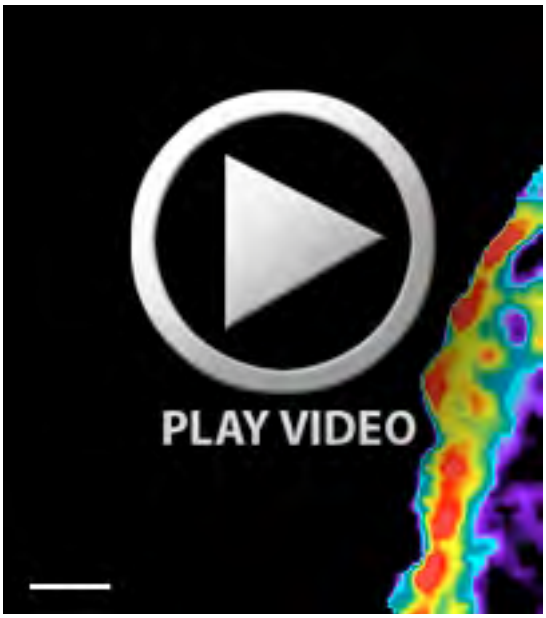


Movie 6. Ratio (FRET/CFP) images of MTLn3 cells. MTLn3 cells expressing the RhoC biosensor and transfected with p190RhoGEF-mut-mCherry were starved for 3 h and stimulated with 5 nM EGF at 37°C (EGF was added in the fourth frame). The images were taken at 10 s intervals. Movie corresponds to Fig. 5C. Total duration: 5 minutes. Dash white lines represent an area of approximately one micron depth from the cell edge. White arrows highlight areas with low RhoC activity behind the leading edge. Scale bars represent 1 μm.

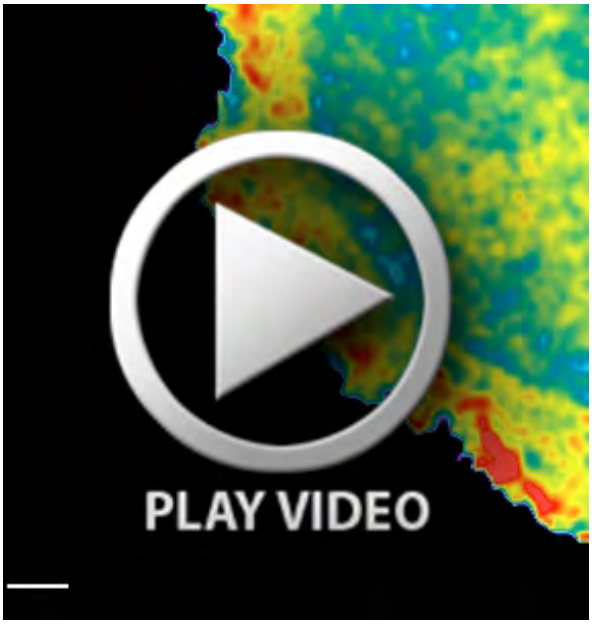


Movie 7. Ratio (FRET/CFP) images of MTLn3 cells. MTLn3 cells expressing the RhoC biosensor and knocked down for p190RhoGAP were starved for 3 h and stimulated with 5 nM EGF at 37°C (EGF was added in the fourth frame). The images were taken at 10 s intervals. Movie corresponds to Fig. 5F. Total duration: 5.5 minutes. Dash black lines represent an area of approximately one micron depth from the cell edge. White arrows highlight areas with RhoC activity at the leading edge. Scale bars represent 1 μm.

Movie 8. MTLn3 cells expressing p190RhoGEF-GFP were starved for 3 h and stimulated with 5 nM EGF at 37°C. The images were taken at 10 s intervals. Movie corresponds to the inset from supplementary material Fig. S4a. Total duration: 5 minutes. Black arrows highlight accumulation of p190RhoGEF. Scale bars represent 10 μm.



Movie 9. Ratio (FRET/CFP) images of MTLn3 cells. MTLn3 cells expressing the RhoA biosensor were starved for 3 h and stimulated with 5 nM EGF at 37°C (EGF was added in the first frame). The images were taken at 10 s intervals. Movie corresponds to supplementary material Fig. S8a. Total duration: 3.8 min. White arrows highlight areas with RhoA activity at the tip of the leading edge. Scale bars represent 2 μm.



Movie 10. Ratio (FRET/CFP) images of MTLn3 cells. N-WASp/Wave 2 depleted MTLn3 cells expressing the RhoA biosensor were starved for 3 h and stimulated with 5 nM EGF at 37°C (EGF was added in the first frame). The images were taken at 10 s intervals. Movie corresponds to supplementary material Fig. S8e. Total duration: 5.6 min. White arrows highlight areas with RhoA activity at the tip of the leading edge. White arrowheads point to areas of high RhoA activity in filopodia-like protrusions. Scale bars represent 2 μm.